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Differential effects of steroids on leukocyte-mediated glomerulonephritis in the rabbit

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Differential effects of steroids on leukocyte-mediated glomerulonephritis in the rabbit. The effects of steroids on the development of injury in two models of experimental glomerulonephritis (GN), (one mediated by neutrophils, the other by macrophages) were compared. The neutrophil-associated lesion [initiated by heterologous antiglomerular basement membrane (GBM) antibody] was characterized by the development of an exudative endocapillary GN with heavy neutrophil accumulation [mean, 6.9 neutrophils/glomerular cross section (N/GCS) ± 2.9 SD], minor macrophage infiltration [7.9 macrophages/glomerulus (M/G) ± 2.2 SD] and heavy proteinuria (1905 mg/24 hr ± 520 SD). Steroid-treated (methylprednisolone, 2 mg/kg/12 hr i.v.) rabbits developed a marked monocytopenia, mild neutrophilia, and significant reduction in glomerular macrophage accumulation (0.3 M/G 0.02 SD). However, neutrophil accumulation (6.1 N/CGS ± 2.5 SD), histological appearances, and proteinuria (1820 mg/hr ± 490 SD) were unaffected. The macrophage-associated model of GN was induced by passive autologous rabbit anti-sheep IgG 15 hr after the injection of a subnephritogenic dose of the same anti-GBM antibody. The glomerular lesion was characterized by a diffuse endocapillary proliferative GN with heavy macrophage infiltration (54 M/G ± 8 SD), insignificant neutrophil accumulation (0.8 N/GCS 0.02 SD), and the regular development of proteinuria (420 mg/24 hr ± 80 SD). Steroid-treated rabbits developed a mild neutrophilia and a significant monocytopenia associated with abrogation of glomerular macrophage accumulation (2.3 M/G ± 0.8 SD). This was associated with the prevention of the development of GN and proteinuria (22 ± 9.5 SD). Thus, steroids produce monocytopenia and prevent glomerular macrophage accumulation and associated injury whereas neutrophil accumulation and injury is unaffected. These data suggest steroids may have widely varying effects on the outcome of leukocyte-associated experimental GN depending on the nature of the infiltrating cells.

Effets différentiels des stéroïdes sur la glomérulonéphrite à médiation leucocytaire chez le lapin. Les effets des stéroïdes sur le développement des lésions ont été comparés dans deux modèles de glomérulonéphrites expérimentales (GN) (l'une médiée par les neutrophiles, l'autre par les macrophages). La lésion associée aux neutrophiles [initialisée par un anticorps hétérologue anti-membrane basale glomérulaire (GBM)] était caractérisée par le développement d'une GN endocapillaire exsudative avec une accumulation massive de neutrophiles [en moyenne, 6,9 neutrophiles/section transversale de glomérules (N/GCS) ± 2,9 SD], une infiltration macrophagique minime [7,9 macrophages/glomérule (M/G) ± 2,2 SD] et une protéinurie massive (1905 mg/24 hr ± 520 SD). Des lapins traités avec un stéroïde (méthylprednisolone 2 mg/kg/12 hr i.v.) ont développé une monocytopénie marquée, une neutrophilie modérée, et une réduction significative de l'accumulation macrophagique glomérulaire (0,3 M/G ± 0,02 SD). Néanmoins l'accumulation neutrophilique (6,1 N/GCS ± 2,5 SD), l'aspect histologique, et le protéinurie (1820 mg/hr ± 490 SD) n'étaient pas affectés. Le modèle de GN associée aux macrophages était produit par injection passive d'IgG de lapin anti-mouton autologue, 15 heures après l'injection d'une dose sub-néphritogène du même anticorps anti-GBM. Les lésions glomérulaires se caractérisaient par une GN proliférative endocapillaire diffuse avec infiltration macrophagique massive (54 M/G ± 8 SD), une accumulation neutrophilique insignifiante (0,8 N/GCS ± 0,02 SD), et l'apparition régulière d'une protéinurie (420 mg/24 hr ± 80 SD). Des lapins traités avec le stéroïde ont développé une neutrophilie modérée et une monocytopénie significative associées à la suppression de l'accumulation macrophagique glomérulaire (2,3 M/G ± 0,8 SD). Cela allait de pair avec la prévention du développement de la GN et de la protéinurie (22 ± 9,5 SD). Ainsi, les stéroïdes produisent une monocytopénie et préviennent l'accumulation glomérulaire de macrophages et les lésions associées tandis que l'accumulation neutrophilique et ses lésions ne sont pas affectées. Ces données suggèrent que les stéroïdes pourraient posséder des effets largement variables sur le devenir des GN expérimentales associées aux leucocytes selon la nature des cellules infiltrantes.

The effect of steroids on the course of human glomerulonephritis is controversial [1–6], and there is no current rational basis for selecting particular types of glomerulonephritis in which they may have potential benefit. However, much is known of their effects on many components of the inflammatory systems thought to be important as mediators of injury in glomerulonephritis. In particular, their effect on circulating leukocytes has been well demonstrated [7–22]. Cells of the macrophage/monocyte system show a particular vulnerability to glucocorticoid administration in therapeutic dose levels. A profound monocytopenia is associated with steroid administration and most of the specialized functions of these cells are depressed. Neutrophil function, however, is relatively refractory to the use of steroids and a neutrophil leukocytosis follows their administration. Thus, it would appear that steroids are of greater potential benefit in macrophage-associated forms of glomerulonephritis. Macrophages have been shown recently to be prominent participants in immunologically induced glomerular damage, both in human disease [23–29] and experimental glomerulonephritis [30–37] Evidence has also been accumulating that they are important mediators of glomerular injury [31–32]. Neutrophils have also been shown to be capable of inducing glomerulonephritis [38] although in different experimental models. It was therefore decided to compare the effects of steroids in neutrophil and macrophage-dependent models of glomerular injury to assess the place for steroid treatment in proliferative glomerulonephritis associated with a significant circulating leukocyte infiltration.

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Methods

Animals. New Zealand white rabbits, weighing 1.6 to 2.5 kg, were used.

Experimental models of injury

Heterologous anti-GBM antibody-induced glomerulonephritis. This was initiated by the single intravenous injection of sheep anti-rabbit GBM antibody (35 μg/g kidney fixing antibody, KFA). Rabbits were kept in metabolic cages for 24 hr and urine was collected for the assessment of proteinuria. After 24 hr the rabbits were sacrificed and the kidneys were removed for subsequent examination.

Passive autologous anti-GBM antibody-induced glomerulonephritis. This model was initiated with a subnephritogenic intravenous dose of the same sheep anti-rabbit anti-GBM antibody used above (6.2 μg/g KFA) followed 15 hr later by passive rabbit anti-sheep gamma globulin serum (4.5 μg/g KFA). Urine was collected over the 24-hour period following the administration of the passive autologous antibody for the assessment of proteinuria. Animals were sacrificed at this time, and the kidneys were removed and assessed as outlined below.

Histological assessment

Portions of kidney cortex were fixed in Bouin's fixative, embedded in paraffin, sectioned at 2 μm, and stained with H & E and periodic acid Schiff's reagent.

Assessment of proteinuria

Proteinuria was assessed by a turbidometric method using a final concentration of 2.5% sulphosalicylic acid [39]. Light transmission was recorded spectrophotometrically (Farrant Optical Co., New York, New York) at 610 nm and the protein concentration determined from comparison with a standard curve made up from bovine serum albumin (BSA).

Leukocyte counts

Monocyte identification was determined by the use of two monocyte specific histochemical stains for the presence of nonspecific esterase: the alpha naphthol acetate method [40] and the alpha naphthol butyrate method [41]. Differential counts were performed on 200 cells stained with each method and the final differential count was expressed as the mean of counts obtained by both methods.

Assessment of glomerular macrophages

The presence of macrophages within glomeruli was assessed by the technique of glomerular cell culture. At the time of sacrifice, portions of renal cortex were removed in a sterile manner, and glomeruli were isolated as previously described [32–34, 42]. The isolated glomeruli were grown in individual drops of tissue culture medium (Modified Eagle's Minimal Essential Medium with 10% fetal calf serum, Commonwealth Serum Laboratories, Melbourne, Australia). After 3 days in culture the number of macrophages emerging from the isolated glomeruli was assessed. At least 10 glomeruli/kidney were assessed and the results were expressed as the mean number of macrophages/glomerulus.

Assessment of glomerular neutrophils

Neutrophils were observed in glomeruli stained with periodic acid Schiff's reagent and cut in equatorial sections. At least 15 glomeruli were assessed per kidney. The results were expressed as the mean number of neutrophils/glomerular cross-section.

Leukocyte depression with nitrogen mustard

Depression of circulating leukocytes was induced by the intravenous injection of nitrogen mustard. An initial dose of 1.7 mg/kg was given followed 36 hr later by 1 mg/kg [32].

Steroid treatment

Methylprednisolone, 2 mg/kg i.v., was administered 30 min prior to the injection of the disease-inducing antibodies. This dose was repeated at 12-hr intervals until sacrifice.

Experimental design

With both experimental models, three groups, each of 12 rabbits were defined: untreated controls, nitrogen mustard, and steroid-treated rabbits. Circulating leukocyte counts were obtained prior to the commencement of each experiment then at regular intervals subsequently. After the rabbits were injected with disease-initiating antibodies, they were placed in metabolic cages and a 24-hr urine collection was obtained to assess proteinuria. At this time the animals were sacrificed. The kidneys were then removed to assess histological appearances and the number of neutrophils and macrophages that had accumulated per glomerulus.

Two further groups of rabbits (each comprised of eight animals) were included to assess the influence of steroids on passive autologous disease induced by increased amounts of passive autologous antibody. In one group a dose of antibody was administered to give a mean kidney binding of 9.0 μg/g and 13.5 μg/g in the second group.

Results

Heterologous phase anti-GBM antibody-induced glomerulonephritis

Untreated rabbits

Leukocyte levels. The injection of heterologous anti-GBM antibody was associated with a minor transient elevation in the levels of neutrophils, lymphocytes, and monocytes. However, the numbers of these cells all returned to the pre-injection range within 4 hr and did not alter during the development of glomerulonephritis (Fig. 1).

Histological appearances. Animals injected with heterologous anti-GBM antibody developed a diffuse exudative proliferative glomerulonephritis (Fig. 2). The most prominent feature of the lesion was the accumulation of neutrophils. Endothelial cell swelling and injury could also be observed at the light microscopic levels.

Glomerular neutrophil accumulation. A mean of 6.9 ± 2.9 SD neutrophils could be observed per glomerular cross section (Fig. 3).

Glomerular macrophage accumulation. A mean 7.8 ± 2.2 SD macrophages were observed per glomerulus at the time of sacrifice (Fig. 3).

Proteinuria. All animals developed heavy proteinuria (mean, 1905 g/24 hr ± 520 SD, Fig. 3).

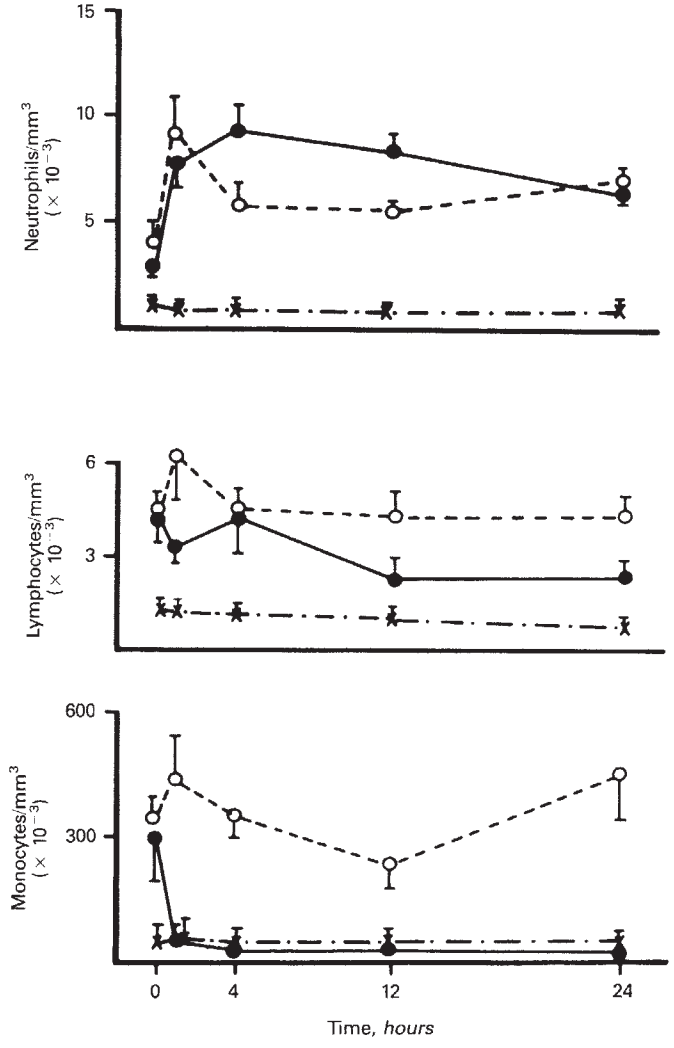

Fig. 1. *The level of circulating neutrophils, lymphocytes and monocytes in* (untreated, ○–○; nitrogen mustard, X–X; and steroid-treated, ●–●) *rabbits developing anti-GBM antibody-induced glomerulonephritis.* All numbers are expressed as mean ± SD.

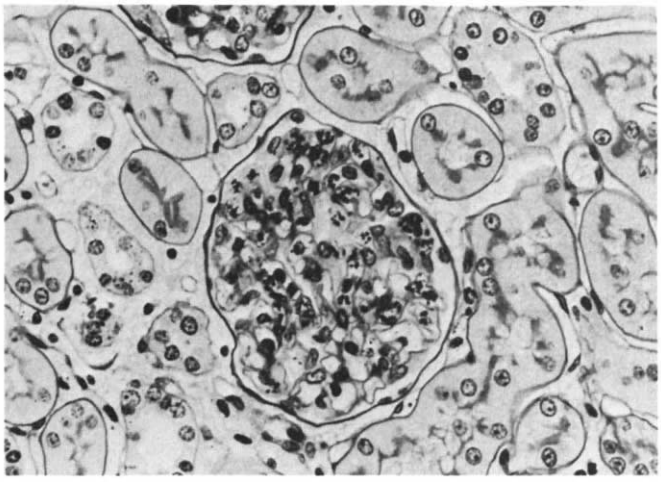

Fig. 2. *Photomicrograph of a glomerulus from a rabbit with anti-GBM antibody-induced glomerulonephritis.* An endocapillary glomerulonephritis with predominant neutrophil accumulation developed is shown. (Original ×120)

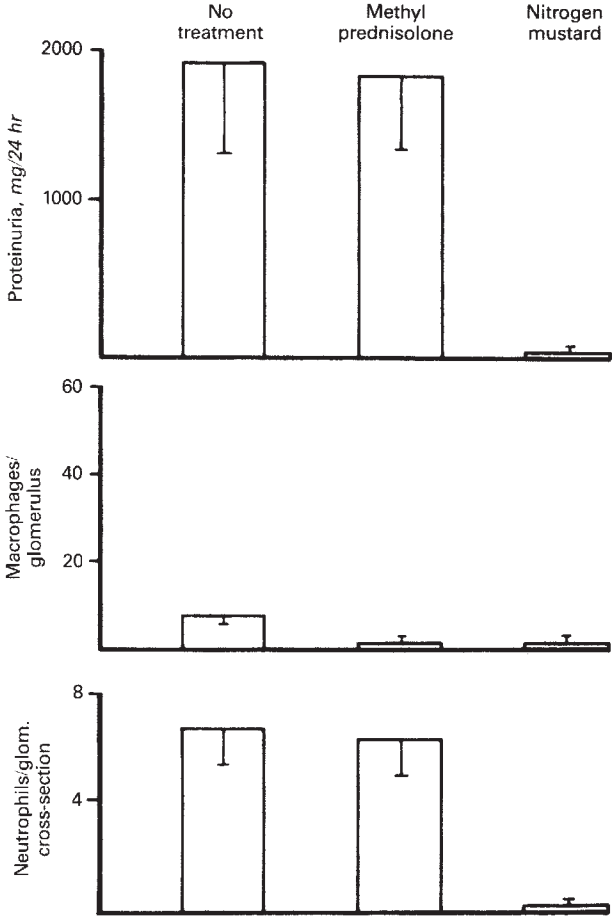

Fig. 3. *The extent of proteinuria and numbers of neutrophils and macrophages accumulating in glomeruli of rabbits developing anti-GBM antibody-induced glomerulonephritis.* All numbers are expressed as mean ± SD.

Nitrogen mustard-treated rabbits

Leukocyte levels. Animals pretreated with nitrogen mustard developed a severe pan-leukopenia (Fig. 1). Levels of neutrophils and monocytes were both reduced to 10% of pretreatment levels. Lymphocytes were also significantly reduced but to a lesser extent.

Histological appearances. Animals pretreated with nitrogen mustard did not develop significant glomerular lesions (Fig. 4).

Glomerular neutrophil accumulation. Nitrogen mustard was associated with almost complete abrogation of neutrophil accumulation (mean, 0.4 neutrophils/glomerular cross-section 0.08 ± SD; $P < 0.05$; Fig. 3).

Glomerular macrophage accumulation. Nitrogen mustard pretreatment prevented the minor macrophage accumulation that was observed in untreated animals (mean, 0.6 macrophages/glomerulus ± 0.07 SD; $P < 0.05$; Fig. 3).

Proteinuria. Animals treated with nitrogen mustard did not develop significant proteinuria (mean, 14 mg/24 hr ± 6 SD; Fig. 3). This was not significantly different from levels seen with normal animals.

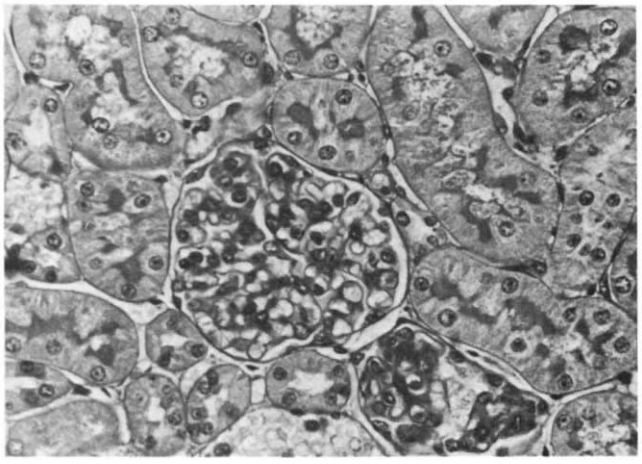

Fig. 4. *Photomicrograph of a glomerulus from a rabbit treated with nitrogen mustard prior to the injection of anti-GBM antibody.* No significant lesion was developed. (Original ×120).

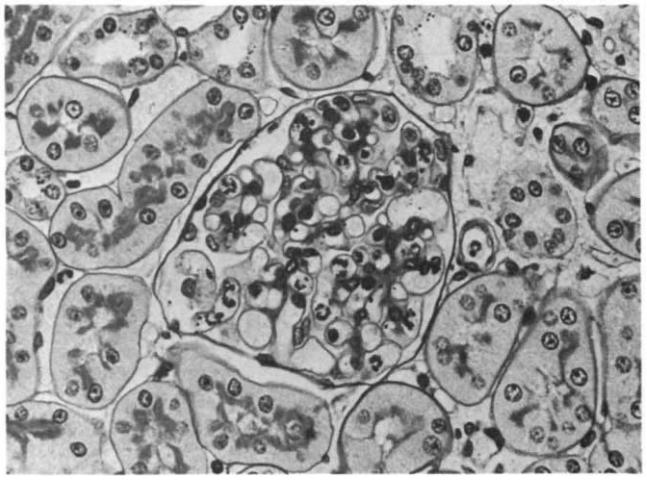

Fig. 5. *Photomicrograph of a glomerulus from a rabbit injected with anti-GBM antibodies and treated with methylprednisolone.* A marked endocapillary glomerulonephritis developed with prominent neutrophil accumulation. (Original ×120)

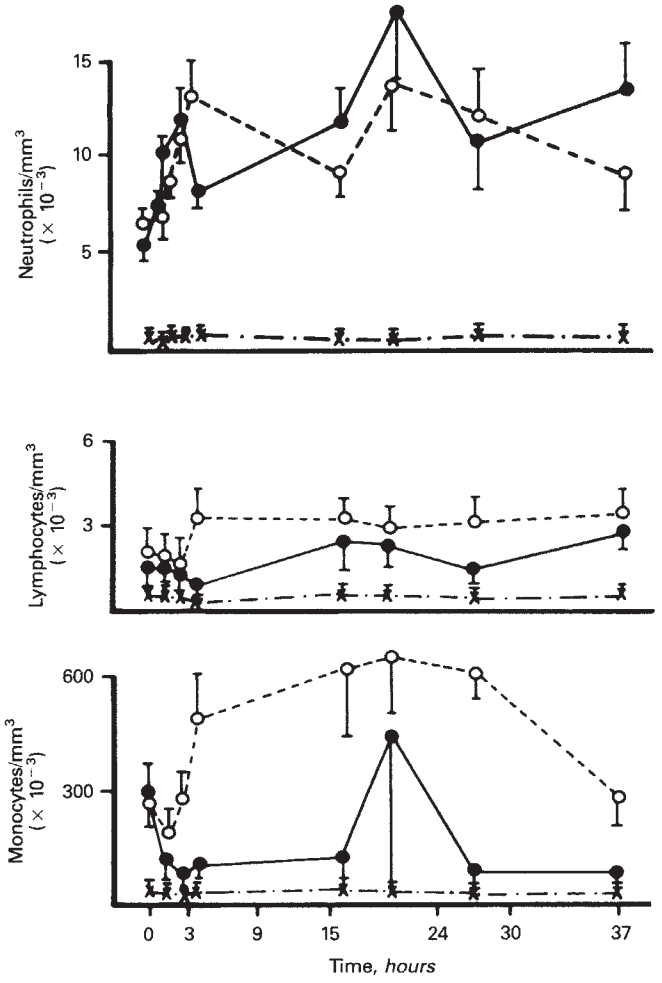

Fig. 6. *The level of circulating neutrophils, lymphocytes, and monocytes in untreated (○–○), nitrogen mustard* (X–X), *and steroid-treated (●–●)animals developing a passive model of the autologous phase of anti-GBM antibody-induced glomerulonephritis.* All figures are expressed as mean ± SD.

Steroid-treated rabbits

Leukocyte levels. The injection of methylprednisolone induced neutrophilia and reduced the levels of both lymphocytes and monocytes. Lymphocytes were least affected while monocyte levels were reduced to less than 10% of pretreatment levels (Fig. 1).

Histological appearances. Steroids had no effect on the histological appearances of the glomeruli of animals injected with heterologous anti-GBM antibodies (Fig. 5). A marked exudative endocapillary proliferative glomerulonephritis exactly similar to that of untreated animals was observed.

Glomerular neutrophil levels. Steroids made no difference to the glomerular accumulation of neutrophils (mean, 6.1 neutrophils/glomerular cross-section ± 2.5 SD, Fig. 3).

Glomerular macrophage accumulation. The minor macrophage accumulation observed in untreated animals was abrogated by the use of steroids (mean, 0.3 macrophages/glomerulus ± 0.02 SD). This was significantly less than the numbers observed in untreated animals ($P < 0.01$) and not significantly different from the levels found following nitrogen mustard treatment (Fig. 3).

Proteinuria. Development of proteinuria was not affected by the use of steroids (mean, 1820 mg/24 hr ± 490 SD).

Passive autologous model of anti-GBM antibody-induced glomerulonephritis

Untreated rabbits

Leukocyte levels. Animals developing the passive autologous model of glomerulonephritis were observed to have a mild transient biphasic neutrophilia associated with the injection of each antibody (Fig. 6). However, the levels of neutrophils soon returned to the pretreatment range. There was a slight but insignificant elevation in lymphocyte counts in this model, but the most significant effect was a marked monocytosis observed throughout the duration of the model (Fig. 6).

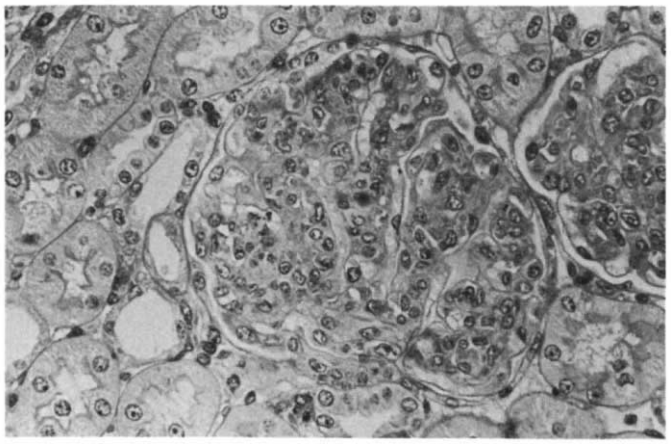

Fig. 7. *A photomicrograph of a glomerulus from a rabbit with passive autologous anti-GBM-induced glomerulonephritis.* A severe endocapillary glomerulonephritis was observed in all animals. (Original ×120)

Histological appearances. Rabbits injected with passive autologous antibody developed an endocapillary proliferative glomerulonephritis. This was a diffuse lesion affecting all glomeruli with the predominant features being a marked increase in the numbers of intracapillary mononuclear cells (Fig. 7).

Glomerular neutrophil accumulation. A mean of only 0.8 neutrophils/glomerular cross-section ± 0.2 SD was observed (Fig. 8). This was not significantly different from the numbers of neutrophils seen in the glomeruli of normal animals (0.4 neutrophils/glomerular cross-section ± 0.1 SD).

Glomerular macrophage accumulation. A marked macrophage accumulation was observed in this model (mean, 54 macrophages/glomerulus ± 8 SD, Fig. 8).

Proteinuria. Significant proteinuria was observed in all animals (mean, 420 mg/24 hr ± 80 SD, Fig. 8).

Nitrogen mustard-treated rabbits

Leukocyte levels. As seen with the heterologous model, pretreatment with nitrogen mustard produced a severe panleukopenia. Again neutrophils was the cell population most affected with a 90% reduction from pretreatment levels while lymphocyte levels were the least affected. A profound monocytopenia was sustained throughout the duration of the experiment (Fig. 6).

Histological appearances. Pretreatment with nitrogen mustard prevented the development of glomerulonephritis (Fig. 9).

Glomerular neutrophil accumulation. Following the use of nitrogen mustard a mean of 0.2 neutrophil/glomerular cross-section ± 0.04 SD was observed (Fig. 8). This was not significantly different from the number of neutrophils observed in the glomeruli of untreated animals developing this type of glomerulonephritis.

Macrophage accumulation. Nitrogen mustard prevented glomerular macrophage accumulation (mean, 0.6 macrophages/glomerulus ± 0.2 SD; $P < 0.05$; Fig. 8).

Proteinuria. Nitrogen mustard-treated animals did not develop significant proteinuria (mean, 12 mg/24 hr ± 4 SD, Fig. 7).

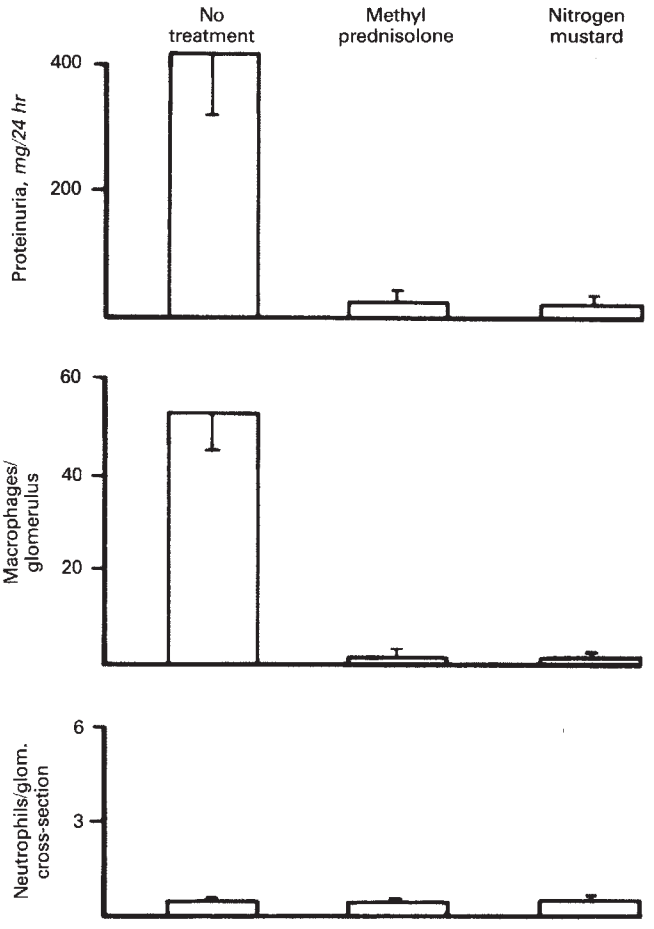

Fig. 8. *The extent of proteinuria and numbers of neutrophils and macrophages accumulating in the glomeruli of rabbits with a passive model of the autologous phase of anti-GBM antibody-induced glomerulonephritis.* All figures are mean ± SD.

Table 1. Effect of steroids in preventing the macrophage accumulation and proteinuria induced by the deposition of increasing quantities of passive autologous rabbit anti-sheep globulin serum

	Control		Steroid-treated	
Antibody-bound, *μg/g*	4.5	4.5	9.0	13.5
Proteinuria, *mg/24 hr*	420 ± 80	22 ± 7.5	38.2 ± 21.2	46.3 ± 18.6
Macrophages/ glomerulus	54 ± 8	2.3 ± 0.8	6.8 ± 2.9	7.2 ± 4.1

Steroid-treated rabbits

Leukocyte levels. Results similar to those seen in steroid-treated rabbits developing heterologous anti-GBM antibody-induced GN were observed. There was a neutrophilia in response to steroid injection while the monocyte levels were profoundly reduced. Lymphocyte levels were only partially reduced (Fig. 6).

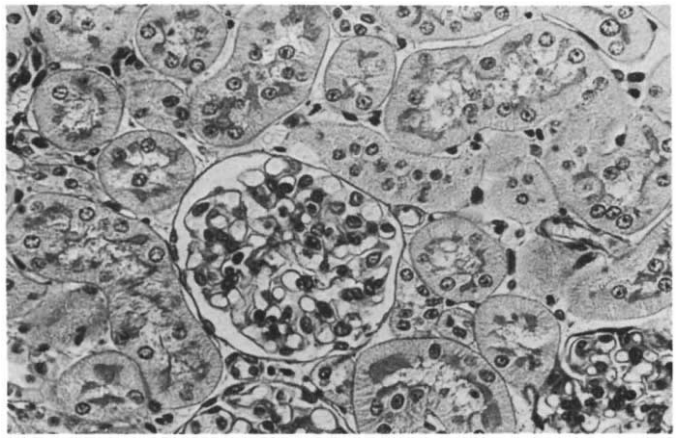

Fig. 9. *A photomicrograph of a glomerulus from a rabbit treated with nitrogen mustard prior to the induction of passive autologous anti-GBM-induced glomerulonephritis.* No significant glomerular lesion developed. (Original ×120)

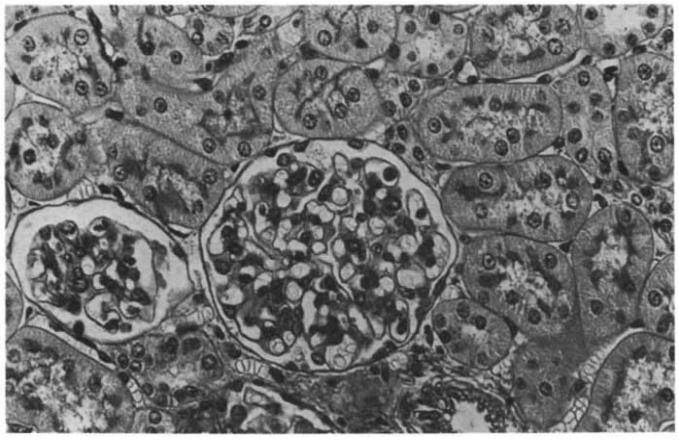

Fig. 10. *A photomicrograph of a glomerulus from a rabbit treated with methylprednisolone during the development of passive autologous anti-GBM-induced glomerulonephritis.* The development of glomerulonephritis was prevented. (Original ×120)

Histological appearances. Steroid-treated animals did not develop a significant glomerular lesion (Fig. 10).

Glomerular neutrophil accumulation. Steroid-treated animals had a mean of 0.6 neutrophils/glomerular cross-section ± 0.2 SD (Fig. 8).

Glomerular macrophage accumulation. Steroid usage was associated with a significant reduction in the levels of glomerular macrophages (mean, 2.3 macrophages/glomerulus ± 0.8 SD; $P < 0.05$; Fig. 8).

Proteinuria. Steroid-treated animals did not develop significant proteinuria (mean, 22 mg/24 hr ± 7.5 SD; $P < 0.01$; Fig. 7).

The effect of steroids on injury induced by increasing quantities of passive autologous antibody

Steroid treatment still afforded substantial protection from injury when increasing quantities of disease induced passive autologous antibody were administered (Table 1). An increase in the extent of proteinuria and macrophage infiltration was observed with higher levels of kidney-bound antibody, but these were all significantly less than that observed in untreated rabbits given the lowest level (4.5 μg/g) of passive autologous antibody (all values $P < 0.05$).

Discussion

Recent studies have shown that neutrophils and macrophages are important mediators of injury in experimental glomerulonephritis [31, 32, 38]. As steroids are known to profoundly influence macrophage function, it was felt likely that these agents may abrogate macrophage-mediated glomerular injury. The current experiments strongly support this hypothesis. Neutrophil function is more resistant to steroid action, and neutrophil-induced glomerular injury was found to be unaffected by steroid therapy.

The two experimental models of glomerulonephritis studied proved suitable to compare the effects of steroids on neutrophil- and macrophage-mediated injury. Injury did not occur in either model in rabbits with nitrogen mustard-induced panleukopenia, confirming that injury depended on circulating leukocytes. In the heterologous anti-GBM antibody-induced model the predominant infiltrating leukocyte was the neutrophil with only a minor macrophage accumulation.

Unlike neutrophils (easily recognized by routine light microscopy), macrophages do not have unique cytological features to allow their participation in glomerular injury to be assessed by routine histology. Therefore, the technique of glomerular cell culture, previously shown to be capable of affording a semiquantitative evaluation of macrophage accumulation, was employed [32, 34, 36]. As neutrophils do not readily survive in tissue culture, they cannot be evaluated by this technique so two different methods of assessing leukocyte accumulation were necessary.

The two models were chosen for study because there was clearly a major difference in the nature of the infiltrating cells. In fact, the models were deliberately contrived to produce these results so that the effect of steroids on injury induced by these two cell populations could be compared.

Heterologous anti-GBM antibody is known to produce injury by complement activation and consequent neutrophil attraction [38]. It is also known that a threshold (the nephritogenic threshold) quantity of antibody must be bound before injury occurs [38]. In the macrophage-related model of injury a subnephritogenic dose of antibody was used so that neutrophil accumulation would be minimal. Macrophage accumulation is far greater in the autologous phase of injury than the heterologous phase [33]. This fact was exploited to augment macrophage accumulation in the passive model by injecting passive autologous rabbit antibody. A previous study has confirmed that this same lesion is due to macrophages [32]. A more recent dissection of this model has shown that immune adherence to the Fc portion of the autologous disease inducing IgG is the major mechanism of macrophage accumulation [43]. Complement has been shown to play no role in this lesion [32]. The difference in effect of heterologous anti-GBM antibody and passive autologous antibody in attracting different inflammatory cells is thus likely to be due to the greater potential for the autologous antibody to facilitate autologous immune adher-

ence of macrophages while the heterologous antibody initiates complement-mediated neutrophil ingress.

Steroids are known to affect both antibody production and cell-mediated immunity, but, in the short-term, passive models studied no potential for either of these mechanisms exists. Thus, in these experiments the effect of methylprednisolone on macrophage and neutrophil mediation of glomerular injury could be directly compared.

It is possible that the protection from injury afforded by steroids in the passive autologous model is related to the quantity of antibody binding to the kidney. However, despite substantial increases in the levels of injected passive antibody significant protection from both proteinuria and macrophage ingress was observed. A trend toward greater macrophage infiltration and proteinuria was observed suggesting that either the macrophage mediation system may become less responsive to steroids under these conditions or that other steroid-independent mediators may be brought into play.

A limitation of both models is the short duration of injury. This was deliberately contrived so active immune responses would be avoided, but it meant that studies of the effects of steroids on established disease could not be usefully carried out as many animals lost their proteinuria spontaneously after the initial 24-hr collection period.

Steroids were found to have a significant effect on the levels of circulating leukocytes. The well known neutrophilia that is associated with their use was confirmed in both experimental models. The predominant suppressive effect of steroids was on the levels of circulating monocytes with a minor reduction in lymphocytes. Similarly, steroid administration prevented the accumulation of neutrophils. These data provide strong evidence for studying the origin of the infiltrating leukocytes in glomerulonephritis. The observations suggest that experimental macrophage-associated glomerular injury is susceptible to steroid administration while neutrophil injury is refractory. These results do not negate other possible actions of steroids in glomerulonephritis, particularly on antibody production and potential lymphocyte-directed delayed hypersensitivity involvement.

The controversial reports of the effectiveness of steroids on human proliferative glomerulonephritis may relate to the relative participation and steroid sensitivity of each of the individual inflammatory mediator systems in differing forms of human glomerulonephritis. Steroids are known to affect human leukocytes in a manner similar to that seen in the current studies. Systemic administration induces a monocytopenia with an associated neutrophilia [8–12]. It is also known that human neutrophil function is relatively unaffected by steroids while cells of the monocyte/macrophage series appear particularly vulnerable to this drug [7–22]. Steroids inhibit the response of monocytes to several chemotactic factors in vitro [15, 19] and depress their normal bactericidal and fungicidal activity in culture [13, 14, 20]. They antagonize the effect of macrophage migration inhibition factor on monocytes [15, 19] and repress reticuloendothelial clearance of both opsonized and non-opsinized materials [16] and lead to decreased accumulation of monocytes in inflammatory sites [7, 18]. Other important effects include the in vitro inhibition of monocyte IgG and complement receptors in a dose-response fashion [21] and a marked decrease in the secretion of collagenase, elastase, plasminogen activator, and neutral proteases [22].

The current data suggest that in the future the study of human renal biopsies should be expanded to include a profile of the infiltrating leukocytes and that specific pharmacological agents may have relative indications depending on which inflammatory pathways are involved. Macrophage-induced proliferative glomerulonephritis may be potentially susceptible to intervention with steroids.

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